ON THE RÔLE OF BACTERIA IN THE BIOLOGICAL METHODS OF SEWAGE PURIFICATION, WITH SPECIAL REFERENCE TO THE PROCESS OF DENITRIFICATION[1].

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CONTENTS.

Introductory.

THE investigations described in the present paper were carried out in connection with the experimental plant laid down by the Corporation

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of Belfast with a view to discover the most suitable method of dealing with the sewage of that city. The necessity of some form of sewage purification arises in Belfast in connection with what is known as the "foreshore nuisance" in Belfast Lough.

At the present time the sewage of Belfast is collected in two main sewers, a low level and a high level. At a first pumping station the whole sewage is collected in a reservoir which is discharged, during ebb-tide only, by a main outfall sewer carried well out into the Lough.

The upper reaches of the Lough are shallow, and at low tide large areas of mud, known as "sloblands" are exposed. On these mud banks there occurs during Spring and Autumn an enormous growth of green sea-weeds, chiefly *Ulva latissima* and various species of *Enteromorpha*. During late Summer and Autumn these sea-weeds are thrown up by storms on the shores of the Lough in large quantity where they decompose and give rise to a most disagreeable and penetrating stench which seriously affects the amenities of the whole surrounding district and no doubt has a deleterious influence on the health of those who are obliged to live in it. Letts (1900, 1901) has shown, by several different lines of argument, that the growth of the green sea-weeds in question is dependent on sewage contamination. *Ulva latissima* contains an unusually high percentage of nitrogen (6 % of the dried weed) and absorbs ammonia with great rapidity from sea-water when added either in the form of ammonium salts or as sewage. Whether these green sea-weeds utilise oxidised nitrogen in the form of nitrates to any large extent has not been definitely proved, but it appears generally to have been taken for granted that this form of nitrogen, which is the typical nitrogenous food supply of other plants, is also available for their growth.

The problem before the Belfast Public Health Committee therefore is, so to treat the sewage as to produce an effluent which has a minimum food value for the *Ulva* and similar sea-weeds, that is to say, an effluent containing a minimum of ammonia and as little nitrate as possible. It will be seen therefore that the object in view is rather different from that in most other towns where a highly nitrated effluent is considered desirable.

The experimental installation consisted in the first place of brick and coke "bacteria beds" for double contact treatment. Prof. Letts undertook the chemical examination of the sewage and effluents while Prof. Lorrain Smith investigated the matter from a bacteriological point of view. The purification effected by these beds averaged 57 % on the

total unoxidised nitrogen as estimated by Kjeldahl's method; this it will be observed is rather a low figure, and no nitrates or nitrites appeared in the effluents. Lorrain Smith (1901) showed that there is a constant decrease in the numbers of bacteria present in the effluent as compared with those in the original sewage, and that the beds which gave the best results chemically showed the largest reduction in bacteria. He also showed that the reduction in number of the bacteria was not due to exhaustion of the necessary pabulum in the sewage but that there must be other factors at work in the beds which lead both to the disappearance of nitrogen and to the destruction of the bacteria. Flasks of diluted nutrient broth inoculated with a loopful of sewage showed after five days a reduction of the nitrogen (Kjeldahl) of at most 12 %, while sometimes the results were entirely negative. On the other hand, when the same broth was added to bricks removed from the contact bed a very striking disappearance of nitrogen occurred, amounting in some cases to 70 % in three days. The bricks used were found to be covered with a sediment consisting largely of vegetable and animal organisms, and a copious layer of a similar sediment formed at the bottom of the flasks. This on analysis was found to be very rich in nitrogen. (It is clear that in these experiments processes of "adsorption" played a large part; this will be referred to later.) Lorrain Smith concluded that this layer of sediment on the bricks, consisting largely of animal and vegetable forms of life, was essential to the efficient working of the beds, and that a certain proportion of the nitrogen disappearing was built up into the bodies of these organisms. There was thus taking place in the beds a cyclical change in the forms of life such as occurs in a decomposing organic fluid, the later forms causing a more or less complete extermination of those appearing earlier in the series. "In this cycle of living forms, bacteria have an early place, owing, no doubt, to their power of rapid growth, and their turn to be exterminated comes correspondingly early also. In the contact beds a great process of extermination takes place. That this extermination is on a large scale may be inferred not only from the observed reduction in numbers, but also conversely from the overwhelming increase in numbers which appears in all the samples if they are kept in conditions favourable for bacterial growth. The effluent then represents the typical organic fluid at the point in the cycle of events when the bacterial forms are being exterminated. It is easy on this hypothesis to understand why the ratio of extermination of the bacteria should be in direct relation to the percentage of purification.

The bacteria, we may suppose, have absorbed into their bodies the greater proportion of the nitrogen available for food. They become in turn the food of infusorians which live in and form the sediment on the bricks, and so the change in forms of life proceeds. The cycle reached its final stage so far as I was able to observe with the introduction of worms (Oligochaeta). These would ultimately pass out to the Lough and become the food of fishes. The nitrogen by this indirect means passes away from the beds in the form of animal tissue. All the nitrogen which can be diverted to this end vanishes from solution, and the sewage is purified in proportion. To strike the cycle and measure the magnitude of one of its events is to measure the general capacity of this living economy for dealing with the available food nitrogen at any given stage, or indeed at all stages of its existence. To measure the ratio of extermination of bacteria therefore, is to measure the percentage of purification. Hence the correspondence between the two ratios." (Prof. Lorrain Smith's *Report.*)

On leaving Belfast in 1904 Prof. Lorrain Smith asked me to continue investigations on the bacteriology of the sewage and the effluents from the contact beds which had then been constructed on a larger scale, in order that bacteriological results might be available for comparison with the chemical analyses made by Prof. Letts.

It was desired in particular to determine to what extent the reduction in the numbers of bacteria is a measure of the chemical purification effected, and whether the different classes of bacteria are destroyed to the same extent. Later, a rotary "sprinkler" was constructed which gave a highly nitrated effluent. In connection with this, at Prof. Letts' suggestion, small experimental contact beds were used which were supplied with mixtures in varying proportions of the nitrated effluent from the sprinkler and the septic tank effluent. In these beds a large proportion of the oxidised nitrogen disappeared; they were therefore known as "denitrifying beds." This led to an investigation of the denitrifying bacteria in sewage and of their chemical action on nitrates in order to determine whether it would be possible to get rid of the nitrates in the effluent by their means. The combination of the sprinkler, which gives a highly nitrated effluent, with contact beds in which denitrification occurs, has thrown fresh light on the fate of the nitrogen which disappears in the process of purification by contact bed treatment.

There can now be no doubt that in this process a considerable amount of nitrogen escapes in gaseous form and an endeavour has

been made to get some idea of the relative proportion in which this occurs.

The present investigation therefore may be divided into two parts, viz.:

(1) An examination of the experimental contact beds, and of the sprinkler, with reference to the disappearance of certain groups of bacteria.

(2) An investigation of the occurrence of denitrification in sewage with reference to the bacteria concerned in the process, with isolation in pure culture of certain of these bacteria and a study of their chemical effects upon nitrates.

PART I.

(1) *The reduction in bacteria and its relation to chemical purification.*

The first series of observations were conducted during July and August, 1904, and consisted of an examination of the large double contact beds A, B, D, F and G. These were made up as follows:

Beds A:	Upper:	Medium Brick.
	Lower:	Fine ,,
Beds B:	Upper:	Medium Clinker.
	Lower:	Fine ,,
Beds D:	Upper:	Medium Coke.
	Lower:	Fine ,,
Beds F:	Upper:	Coarse Limestone.
	Lower:	Medium ,,
Beds G:	Upper:	Medium Limestone.
	Lower:	Fine ,,

"Coarse" signifies passed by $2\frac{1}{2}$ in. mesh not by $1\frac{1}{2}$ in. mesh.
"Medium" ,, ,, $1\frac{1}{2}$,, ,, $\frac{1}{2}$,,
"Fine" ,, ,, $\frac{1}{2}$,, ,, $\frac{1}{8}$,,

The screened and sedimented sewage was treated in an open "septic tank" where it remained for about six hours and then on the contact beds, the period of each contact being three hours.

Full details of the structure and method of working these beds and of the other plant referred to in this paper are given in Prof. Letts' *Third Report* (1908).

The samples of sewage at the various stages were taken as far as possible so that they are strictly comparable with one another. For

example, on August 5th a series of samples labelled as follows was received.

	Date	Time	
No. 1.	Aug. 4th	3.30 p.m.	Crude Sewage.
No. 2.	Aug. 4th	7.30 p.m.	Screened and Settled.
No. 3.	Aug. 5th	1.0 a.m.	Septic Tank Effluent.
No. 4.	Aug. 5th	7.0 a.m.	A. Lower Bed Effluent.
No. 5.	Aug. 5th	7.0 a.m.	B. ,, ,, ,,
	etc.	etc.	

A similar set of samples was examined each time. The samples were received at the laboratory at 10 a.m. and the examination begun at once. Five examinations were made of this series on the following dates: July 22nd and 26th, August 5th, 12th, and 23rd. The following groups of bacteria were estimated:

(1) Those growing on Gelatin at 22° C.
(2) Those growing on Agar at 37° C. (three examinations).
(3) Spores growing on Gelatin at 22° C. (aërobic).
(4) *Bacillus coli.*
(5) *Streptococcus.*
(6) *Bacillus enteritidis sporogenes* (Klein).

Attention was paid to the three last named bacteria, first, because their presence is easily detected, and secondly, because *B. coli* and *Streptococcus* are characteristic intestinal organisms while *B. enteritidis*, as an anaërobic spore-bearing bacillus, represents a class not otherwise taken into account in the investigation. The first three groups were counted by the ordinary plate methods. The dilutions were made by means of pipettes delivering 1 c.c., flasks of about 250 c.c. capacity containing 100 c.c. of water, and test-tubes containing 9 c.c. of water. A large supply of these in a sterile condition was prepared before each examination of a series of samples. The flasks enable the high dilutions required to be rapidly attained, and have the further advantage of allowing very thorough shaking so as to secure a uniform distribution of the bacteria. For the counting of spores the tubes containing the necessary dilutions were exposed to a temperature of 80° C. for ten minutes to kill off the other forms.

After some preliminary examinations it was found that the dilutions required to give satisfactory plates for enumeration were as follows:—

Crude Sewage, Screened Sewage, and Septic Tank.

For gelatin plates:	1/1,000,000	and	1/10,000,000.
For agar	,,	1/100,000	,,	1/1,000,000.
For spores	,,	1/10	,,	1/100.

Contact Bed Effluents.

For gelatin plates:	1/100,000	and	1/1,000,000.
For agar	„	1/10,000	„	1/100,000.
For spores	„	1/10	„	1/100.

Two plates were made with 1 c.c. each of each dilution, and where possible only plates with more than five and less than a hundred colonies were counted. The agar plates were incubated at 37° C. for 24 hours; the gelatin plates at 22° C. for two days.

B. coli and streptococci were estimated by the following method which is a modification of that described by Prescott and Winslow (1904) and depends upon the fact that when these two organisms are grown together in glucose broth, as the medium becomes acid the streptococci outgrow the bacilli and after two days can be easily isolated from the mixture by means of litmus lactose agar plates. On this medium the streptococcus colonies are small and intensely red, and are easily distinguished from the larger and less acid colonies of *B. coli*. One cubic centimetre of the several dilutions of the sewage was added to glucose broth in Durham's fermentation tubes. The tubes were incubated at 37° C., and on the following day those which showed gas formation were noted, and a loopful from each was smeared on the surface of a gelatin plate, the tubes being returned to the incubator. After two days at 22° C. these gelatin plates were examined and non-liquefying colonies (where possible those showing the typical "vine-leaf" structure) were picked out and tested in pure culture. The additional tests used for the identification of *B. coli* were, gas formation in glucose broth, coagulation of milk, and indol formation in peptone water.

For streptococci the same glucose broth tubes were examined after incubation at 37° C. for two days. It was usually sufficient to examine a loopful in a hanging drop preparation under the microscope for the presence of chains of streptococci.

The "*B. enteritidis* test" was done as follows: 1 c.c., 1/10 c.c., and 1/100 c.c. of the samples were added to deeply filled whole milk tubes which were first heated to 80° C. for ten minutes and afterwards incubated at 37° C. for twenty-four hours. Clotting of the milk, with gas formation and tearing of the clot, were regarded as evidence of the presence of organisms of this class.

Of course these methods only give a rough approximation to the

number of bacteria of the classes named which are present; thus if the presence of *B. coli* be demonstrated in 1/100,000th of a cubic centimetre, but not in a millionth, we may conclude that there are at least 100,000 but not so many as a million present per c.c. and in the tables the fraction 1/100,000 is used to indicate this. If a number of tubes be inoculated from the same sample a closer approximation may be obtained; thus if *B. coli* be found in 5 tubes out of 10 inoculated with 1/100,000 c.c. we conclude that the number present in 1 c.c. is about 500,000. An approximation to the average number in a large series of examinations may be made in the same way, and the averages for *B. coli* and streptococci given in Table I are calculated on this principle.

The detailed results of the examination of each sample are given in Table I, and the average for each sample noted for the bacteria growing on gelatin and on agar and for spores.

The proportion of bacteria growing at body temperature to those growing at 22° C. is nearly 1 to 10 in the untreated sewage and in the effluent from bed A, while it is as 1 to 5 in the other effluents.

The spores are remarkably few in number, varying from 2000 per c.c. in the crude sewage to 90 per c.c. in effluent A. This may be taken to indicate that the sewage is a favourable medium for bacterial growth.

The presence of *B. coli* and streptococci was demonstrated in nearly every case in 1/100,000 c.c. and occasionally in 1/1,000,000 c.c. both in the untreated sewage and in the effluents.

The constant decrease in the numbers of *B. enteritidis* during the period of observation is remarkable. On July 22nd this organism was present in 1/1000 c.c. of the crude sewage, while on August 23rd it could only be found in 1 c.c.

A calculation of the average numbers of *B. coli*, streptococci, and *B. enteritidis*, is made for the three samples of untreated sewage, and for all the effluents respectively. The actual numbers given are probably too high, but they serve to indicate the percentage reduction for these classes.

There is a progressive diminution in the numbers of bacteria during the whole process of purification; they are reduced to about one half in the screened and settled sewage, and again to about one half in the "septic tank." It should be noted in connection with this latter fact, that the sewage was not subjected to a closed septic tank treatment. The tank was open, and the process took more the form of a further sedimentation than anything else.

The greatest reduction in numbers of bacteria occurs in the contact beds.

This is best seen in Table II, where the percentage reduction in the various classes is shown, calculated on the crude sewage, and also (for the effluents) on the septic tank.

The corresponding chemical results (average of two examinations on July 22nd, and July 26th, by Prof. Letts) are given for comparison.

It will be observed that the effluent from bed A, which shows the best chemical results, especially as regards loss of free ammonia, also shows the greatest reduction in bacteria of all classes. The various beds may in fact be arranged in the same order of efficiency whether they are judged by the chemical or by the bacteriological standard. There can be no doubt therefore that the decrease of bacteria in the effluent is an exact measure of the chemical efficiency of a contact bed. The superiority of bed A is most marked when judged by the destruction of bacteria growing at 37° C. and of spores. It would appear therefore that most attention should be paid to these groups in measuring the efficiency of a bed by bacteriological methods.

With regard to *B. coli* and *Streptococcus* it should be noted that these are proportionately less reduced in number than the other groups; and if these organisms be taken to represent the pathogenic class, there is no evidence in the above results to show that this class is destroyed in the "bacteriological" methods of sewage purification, as has sometimes been supposed.

With reference to the cause of the disappearance of the bacteria shown in the above enumerations it must be observed that the reduction begins in the sedimenting sewage and continues in the so-called "septic tank." It appears probable that here the process is a merely mechanical one, due to sedimentation, for we can hardly believe that higher forms of life are at work destroying the bacteria under such conditions. But the reduction in numbers of bacteria in the same period of time is much greater in the contact beds. This fact is important, and affords clear evidence that other than purely mechanical factors are at work in causing the disappearance of bacteria in the beds. That these factors are essential to the efficient working of the beds is shown by the correspondence of the chemical and bacteriological results.

Included in Table II are the results of one examination of the effluent from the rotary sprinkler, this effluent being in every way comparable with those from the contact beds. The reduction in bacteria is much greater than that shown by the best of the contact

beds. This is in agreement with the results of the next series of examinations, which will now be given.

This consisted in a bacteriological examination of the effluent from the sprinkler and from the denitrifying contact beds used in connection with it, as compared to the septic tank effluent. Six observations were made during January and February, 1905.

The sprinkler was constructed in segments of different materials corresponding to those used in the contact beds already described. It was worked intermittently by an automatic arrangement and was supplied from the septic tank. The denitrifying beds were of small size, were made up of bricks similar to the A beds, and were worked in a similar fashion. They were supplied with a mixture of equal parts of the sprinkler and septic tank effluents during the period of the earlier examinations. On the dates of the two last examinations they were receiving the sprinkler and septic tank effluents in the proportion of 3 : 4. The lower denitrifying bed for second contact was started early in February and was only examined on two occasions.

The same technique was employed as before, and the same groups of bacteria were estimated (with the exception of spores and *B. enteritidis*).

Corresponding chemical analyses, made by Prof. Letts, are available for the whole series, and are given in Table IV. The detailed results of the examinations on the different dates are given in Table III, and also the averages for the whole series. The number of bacteria in the mixture of sprinkler and septic tank effluents are calculated for each date, and given in the table for comparison with the effluents from the denitrifying beds. Table V shows the percentage reduction in the number of bacteria and the percentage chemical purification calculated for the septic tank, and in the case of the denitrifying beds, for the mixture of septic tank and sprinkler effluents.

In comparing the results with those of the first series it must be remembered that in this case the crude sewage was not examined, and the results calculated for the septic tank in the first series must be taken for comparison.

The numbers of bacteria are rather lower than in the first series, corresponding, no doubt, to the different season of the year. The proportion of those growing at 37° C. to those growing at 22° C. is, as before, about 1 : 10, both in the sewage and in the effluents. There is again a general correspondence between the chemical and the bacteriological results. In the sprinkler effluent there is a very large reduction

in the number of bacteria, all the groups being about equally affected. This reduction is considerably greater than that shown by the best of the contact beds (A) in the previous series. The averages are given here for comparison.

Reduction in Bacteria (on Septic Tank).

	22° C.	37° C.
Sprinkler	93·4 %	96 %
A beds	69 %	63 %

This does not correspond to any greater efficiency of the sprinkler from a chemical point of view, as is shown by the following figures.

Chemical Purification (on Septic Tank).

	Free NH_3	Alb. NH_3	Oxy. abs.
Sprinkler	71·5 %	51 %	63 %
A beds	89 %	53 %	72 %

On the other hand there is very little reduction in the bacteria in the upper denitrifying bed, although there is considerable chemical purification (Table V).

These contrasted results show that the reduction in bacteria is not a measure of the chemical purification when processes so different as those at work in the sprinkler, in the denitrifying bed, and in an ordinary contact bed, are in question. This is what we might expect when we remember that the destruction of bacteria is not an essential factor in the purification, but only a usual accompaniment, and an indication of the activity of higher forms of life.

If however we compare the effluent from the lower denitrifying bed with that from the A beds of the first series, we find that the chemical and bacteriological results are practically similar.

Reduction in Bacteria.

	22° C.	37° C.
A beds	69 %	63 %
Denitrifying bed	72 %	87 %

Chemical Purification.

	Free NH_3	Alb. NH_3	Oxy. abs.
A beds	89 %	53 %	72 %
Denitrifying bed	76 %	55 %	67 %

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The amount of nitrate in the two effluents also corresponds (about 0·25 parts N. per 100,000).

This gives us grounds for believing that, on the whole, the same processes are at work in the double contact beds on the one hand, and in the combination of sprinkler and denitrifying beds on the other. It will be shown in the second part of this paper that in the process of denitrification nitrogen escapes into the atmosphere in the form of gas. The facts now before us indicate that in the process of treatment by double contact successive stages of nitrification and denitrification occur, exactly comparable to the stages represented by the sprinkler and the denitrifying beds. When the contact beds are empty, in the so-called "resting" stage, active oxidation takes place, and among other changes of a similar nature, ammonia is oxidised to the form of nitrate. When the bed is again filled, it acts as a "denitrifying bed," and a large proportion of the oxidised nitrogen disappears in gaseous form. This view is confirmed by a general consideration of the physical and chemical processes which occur in "contact beds" and in "percolating beds" (such as the sprinkler). It will be convenient to give here a short account of our present knowledge of these processes.

(2) *Physical and chemical processes in "contact" and "percolating" beds.*

We owe our knowledge of this subject chiefly to the work of Prof. Dunbar, Director of the Hygienic Institute in Hamburg. Dunbar (1900) has shown that the purification of sewage by the artificial biological methods depends very largely on processes of "adsorption." This term is used to indicate a little understood process of a physical nature whereby organic substances in solution are thrown out of solution, or retained in a more concentrated form, in the interstices, or on the surface of solid substances of a suitable nature, when these are immersed in the solution in question.

Thus if pieces of filter paper are placed in a dilute watery solution of a stain, they take up more of the colouring matter than of the water, and the solution in consequence gradually becomes less and less coloured. This is a simple but sufficiently accurate illustration of the process of "adsorption." No distinction is here made between "adsorption," which strictly refers only to surface action, and "absorption" in its limited sense, as this latter term is apt to give rise to confusion from its more common and wider use.

Working at the question from an entirely different point of view, Dunbar confirmed Lorrain Smith's view as to the inadequacy of the purely bacterial theories of sewage purification which had previously been in vogue. He showed that the material used in the construction of the beds, e.g. clinker, is possessed of very considerable adsorptive properties, and that these are very much increased by the slimy deposit which forms on its surface in a ripe bed. Dunbar showed further, by means of small experimental beds, that a sudden great reduction in the amount of organic matter in the sewage takes place within five minutes of the filling of the bed, and that this reduction is only slowly increased on further contact. He also demonstrated adsorption effects by the use of methylene blue, the effluent in this case remaining permanently decolourised, whereas bacterial action does not produce permanent decolouration. The adsorptive powers of a bed are very rapidly exhausted unless it is allowed intervals for rest and recovery. Dunbar showed this by the following experiment. A small contact bed was filled with sewage and allowed to stand for one hour; it was then emptied and filled again five times in rapid succession, and the various effluents gave the following results on analysis.

	Purification	Odour
After standing one hour	56·2 %	Earthy.
1st filling	65·0	,,
2nd ,,	66·2	,,
3rd ,,	32·0	Faintly faecal.
4th ,,	27·3	Faecal.
5th ,,	16·5	,,

There was here a rapid falling off in the purification effected after the second filling.

Dunbar also showed the importance of the resting interval by another set of experiments. In these the amount of oxygen taken up from the atmosphere, and the amount of carbonic acid produced in the bed, were estimated, and were found to be much greater when the bed was empty than when filled.

The most remarkable fact about a contact bed is its great power of recuperation when allowed a sufficient interval for rest. Its adsorptive capacity is rapidly re-established, and this recovery is accompanied by active chemical changes largely of the nature of oxidation. From this point of view the bed shows most activity during its so-called resting period.

The organic material in solution is retained in the bed by adsorption, that in suspension is no doubt retained also by physical processes of a

simpler nature; when the bed is emptied and freely exposed to air, the retained organic material is subjected in a concentrated form to the attack of the various forms of life in the bed, and it is owing to this vital activity that the bed is able to regain its absorptive powers. That the activity of these vital processes is greatest when there is a free supply of air is in accordance with the view that it is chiefly the higher forms of life which are at work. No doubt the bacteria also play an important part in breaking down complex organic substances, and in other respects. If these various forms of life are not allowed to perform their scavenging functions the absorptive powers of the bed are speedily exhausted, and purification ceases.

It is probable that some substances in solution in the sewage are retained in the beds by purely chemical means, independently of adsorption. Thus, as Dunbar (1903) points out, sulphuretted hydrogen combines with iron to form sulphide, which is then oxidised to ferric sulphate. This is precipitated by the alkaline sewage as hydrate, in which form it remains in the bed.

It appears probable also that the adsorptive functions of the bed do not cease when it is emptied. The enormous oxidising power of the empty bed is materially assisted by the presence of adsorbed or condensed oxygen which is more potent chemically than ordinary oxygen. Thus Lübbert has shown that dimethylanilin, when in contact with clinker, is oxidised in air to methyl violet. This reaction does not occur on exposure to air under ordinary conditions; it is commonly effected by heating with potassium chlorate.

In percolating beds like the sprinkler these various processes of absorption and oxidation with resulting purification take place simultaneously. It is quite impossible to understand the very rapid effect produced by such beds apart from the adsorption theory. The sewage leaves the bottom of the bed in a purified condition a very short time after its delivery at the top. The organic material has simply been retained in the bed for future purification. If this is satisfactorily effected the adsorptive powers of the bed are not exhausted and the process of purification goes on continuously. The reduction in the number of bacteria must be regarded from a similar standpoint. These are at first simply retained in the bed by a process of filtration. They are there destroyed in large numbers; otherwise the capacity of the bed for acting as a bacterial filter would soon become exhausted.

PART II.

STUDIES ON DENITRIFICATION.

(1) *Introductory.*

It has long been known that nitrates, in contact with certain organic substances, are destroyed with evolution of nitrogen gas and gaseous oxides of nitrogen. This process is known as denitrification. It is in marked contrast to the opposite process of nitrification, whereby nitrates are formed from ammonia, or, under certain conditions, even from free nitrogen. The latter process is essential for plant life; the former if allowed to proceed unchecked would render plant life impossible. Both processes have been shown in recent years to be due to bacterial action. Denitrification has been studied chiefly in connection with agricultural problems. The literature is already very extensive, and cannot here be referred to in any detail. Full references to it are given in an article by Jensen (1904) in Lafar's *Handbuch der technischen Mykologie.* In that article Jensen divides the bacterial processes which are accompanied by a reduction of nitrates, under the following heads:

(1) Reduction of nitrates to nitrites and ammonia.

(2) Reduction of nitrates and nitrites to lower gaseous oxides of nitrogen (N_2O and NO).

(3) Reduction of nitrates and nitrites with evolution of free nitrogen.

With regard to (1) it is well known that a very large number of bacteria have the power of reducing nitrate to nitrite; it is however doubtful whether in any case ammonia is formed by the reduction of nitrate.

It is chiefly with the processes mentioned under (2) and (3) that we are here concerned. Strictly speaking the term denitrification should be confined to (3).

With regard to the production of lower gaseous oxides of nitrogen little work has been done, and no pure cultures have hitherto been described which reduce nitrate to this stage only.

The typical denitrifying bacteria reduce nitrate or nitrite with formation of free nitrogen gas. When pure cultures of these bacteria are grown in peptone broth containing 0·25 % potassium nitrate or nitrite a very characteristic foaming of the culture occurs owing to the production of gas.

In 1886 Gayon and Dupetit isolated two species of denitrifying bacteria but did not describe their characters sufficiently to allow of identification. When one of these was grown in nitrate broth containing asparagin both nitric oxide (NO) and free nitrogen were produced; in the absence of asparagin only free nitrogen occurred.

The first thorough bacteriological investigation we owe to Burri and Stutzer (1895), who isolated from horse dung two bacteria which they called *B. denitrificans* I and II. These were afterwards renamed by Lehmann and Neumann, *B. denitrificans*, and *B. Stutzeri*, respectively. While *B. Stutzeri* could decompose nitrate with gas formation in pure culture, *B. denitrificans* could only do so when accompanied by *B. coli*. The explanation of this fact was given later by Weissenberg (1897), who showed that *B. denitrificans* can attack and decompose nitrite but not nitrate. Since *B. coli*, in common with many other bacteria, has the power of reducing nitrate to nitrite, it is easy to understand how a combination of these two bacteria can attack nitrate and destroy it with formation of free nitrogen.

Since then a number of other denitrifying bacteria have been isolated and described, and it has been shown that certain bacteria previously known, e.g. *B. pyocyaneus*, possess this property.

(2) *Denitrification in Sewage.*

In 1904 Letts showed that when potassium nitrate was added to septic tank effluent in a proportion equivalent to 2·5 parts of nitric nitrogen per 100,000 parts of mixture, the nitrate disappeared in 24 hours, and in four out of eight experiments the theoretical amount of nitrogen could be recovered in the form of free nitrogen and nitric oxide, the latter being present only in small amount. In one experiment where the septic tank effluent had been previously filtered through porcelain the disappearance of nitrate did not occur.

Prof. Letts also found that if equal parts of septic tank and sprinkler effluents were mixed and allowed to stand in a stoppered bottle, the nitrate disappeared in from one to two days. It occurred to him that this process might be hastened if the mixed effluents were treated in a contact bed; for this reason the denitrifying beds already described were constructed, and it was in fact found that the nitrate disappeared almost completely after three hours contact with the bed. This is shown in Table IV. On Jan. 25th, the nitrate was reduced only from 1·32 to 0·82, but at this date the bed was new; on the subsequent

examinations complete disappearance of the nitrate occurred with the exception of that on Mar. 1st, when only 90 % reduction was found, the amount of nitrate in the sprinkler effluent on this date being exceptionally large. After treatment on the lower "denitrifying bed" a certain amount of nitrate again appeared in the effluent.

Although the single observation of Prof. Letts, mentioned above, in which the sewage was filtered through porcelain, appeared conclusive as to the bacteriological nature of the process of denitrification in sewage, it was thought advisable to confirm this by other means. The following experiments were therefore carried out.

Preliminary experiments to show that denitrification is due to bacterial action.

Test tubes and flasks plugged with cotton-wool were used, no attempt being made to secure anaërobic conditions. The temperature, except where otherwise stated, was 37° C. By "nitrate reaction" the Brucin Sulphuric acid test is to be understood; this of course indicates the presence of either nitrate or nitrite.

Sp. = Sprinkler effluent. S. T. = Septic tank effluent.

(1) 5 c.c. Sp. + 5 c.c. S. T. in test tube: complete disappearance of nitrate reaction in 24 hours.

(2) 10 c.c. Sp. in test tube: no diminution in nitrate reaction in 24 hours.

The sprinkler and septic tank effluents were now filtered through porcelain, tested, and found to be sterile.

(3) 5 c.c. filtered Sp. + 5 c.c. filtered S. T.: no diminution in nitrate reaction in 48 hours.

(4) 5 c.c. filtered Sp. + 5 c.c. filtered S. T. + loopful of unfiltered S. T.: complete disappearance of nitrate reaction in 48 hours.

These experiments show that denitrification is due to the action of micro-organisms which do not pass through a porcelain filter.

A dilute peptone broth (1 in 20 of the ordinary nutrient broth) was now prepared with the addition of 0·03 % potassium nitrate. This contained 2 parts of nitric nitrogen in 100,000, or rather more than the average amount of nitrate in the sprinkler effluent.

(5) Two flasks (capacity 250 c.c.) containing 100 c.c. of this dilute nitrate broth each received 1 c.c. S. T. effluent. One flask (A) was kept at 37° C., the other (B) at 22° C.

	Nitrate reaction	
	A	B
After 24 hours	Positive	Positive
After 2 days	Negative	Positive
After 3 days	Negative	Positive
After 4 days	Negative	Negative

This experiment shows that denitrification occurs more readily at the higher temperature, and confirms the previous results as to the bacterial nature of the process.

(3) *The Denitrifying Bacteria isolated.*

The isolation of denitrifying bacteria was carried out in the first place by means of a dilute nitrite peptone broth, corresponding in composition to the dilute nitrate broth mentioned above.

A flask containing 100 c.c. of this dilute nitrite broth was inoculated with 2/10 of a c.c. S. T. effluent. After 24 hours at 37° C., when the nitrate reaction had disappeared, gelatin plates were made; at the same time another flask was inoculated with a loopful from the first, and after 24 hours a loopful from this was inoculated into a third flask, from which gelatin plates were also made. When colonies appeared on the gelatin plates they were isolated and tested in nitrate and nitrite broth. There appeared to be no advantage in carrying on successive inoculations from one flask to another, as more denitrifying colonies were found on the first set of plates than on the second. Two species of denitrifying bacteria were found. These are described below as Nos. 1 and 2.

In the course of further investigations into the number of denitrifying bacteria present in the various samples of sewage, pure cultures were isolated and tested from time to time. *B. pyocyaneus* was thus found on several occasions, and an additional new species, described below as No. 3, was found.

The bacteria isolated may be divided into two groups, according to whether or not they produce gas in 0·25 % potassium nitrate broth. Those which do so belong to the class of typical denitrifying bacteria. All the bacteria isolated were able to reduce nitrate in pure culture; thus bacteria belonging to the class represented by *B. denitrificans* (L. and N.), which are able only to attack nitrite, were not found.

A. Bacteria which produce gas in 0·25 % potassium nitrate broth: the typical Denitrifying Bacteria.

Bacillus No. 1. This bacillus was isolated on more than one occasion. It appears to be identical with *B. Stutzeri* (L. and N.).

It is a motile non-sporing bacillus, resembling *B. coli* in hanging drop and in stained preparations. It does not retain the stain in Gram's method.

Gelatin stroke culture: A hard dry wrinkled growth along the needle track, slightly yellowish in tint. No liquefaction of the gelatin.

Gelatin plate culture: The surface colonies after two days are round or irregular, elevated and crenated at the margin, and so hard and tenacious that it is difficult to obtain subcultures without lifting off the whole colony. The deep colonies are round and not characteristic.

Agar stroke culture: The growth is hard, dry, and adherent, white in colour, and not very characteristic. *Milk* undergoes no change. In *glucose broth* a uniform turbidity is produced and a film forms on the surface. There is no gas formation, but the medium becomes acid. In peptone water *indol* is produced.

In 0·25 % potassium nitrate broth there is diffuse turbidity with film formation on the surface. The characteristic foaming due to gas formation occurs. The same appearances are found in 0·25 % sodium nitrite broth.

This bacillus does not grow under anaërobic conditions in ordinary broth, but if 0·25 % potassium nitrate be added growth and gas formation occur as before.

A culture was made in 0·25 % potassium nitrate broth in a large flask which was filled quite full and tightly stoppered. From this flask a capillary glass tube was led off and the gas produced was collected over mercury. Samples of this gas were kindly analysed by Prof. Letts with the following result:

First 20 c.c.		Second 20 c.c.	
Nitrogen	99 %	Nitrogen	98 %
Carbonic acid	1 %	Carbonic acid	2 %

This bacillus therefore decomposes nitrate with the formation of free nitrogen.

The only other bacillus belonging to this group which was isolated was *B. pyocyaneus.* This produced the typical foam formation in

0·25 % potassium nitrate broth, but the gas produced was not analysed. Its other characters need not here be described.

B. Bacteria which cause the disappearance of the nitrate reaction, but do not produce gas in 0·25% potassium nitrate broth: B. hyponitrosus group.

Bacillus No. 2. This is a slender, slightly curved bacillus. In broth culture it forms short filaments which show a sluggish serpentine movement. It does not form spores, and it retains the stain in Gram's method. It liquefies gelatin slowly and irregularly; thus a gelatin slope culture shows a white layer along the track of the needle with deep pits produced by liquefaction especially at the lower part of the stroke.

Gelatin plate culture: The colonies appear after 2 days at 22° C. At first there is no sign of liquefaction, the surface colonies forming round white discs about 2 mm. in diameter, and the deep colonies uncharacteristic small spheres. After about 4 days liquefaction begins and proceeds rapidly around all the colonies.

Agar stroke culture is uncharacteristic, somewhat resembling that of *B. coli.*

Milk is coagulated in 7 days at 37° C. There is marked production of *indol* in peptone water. In glucose broth this bacillus does not grow readily. In a fermentation tube containing glucose broth diffuse growth occurs in the open limb of the tube, but none in the closed limb; some acid is produced but no gas.

In 0·25 % potassium nitrate broth a copious turbid growth occurs. No gas is produced, but the nitrate reaction is largely diminished in amount. In dilute nitrate broth the nitrate reaction disappears in 2 days at 37° C.

This bacillus was not identified and its chemistry was not further studied. Its action on nitrates was not so energetic as that of the next bacillus (No. 3) and it appeared to be better to choose the latter as an example of the group for further study from a chemical point of view.

Bacillus No. 3. This bacillus was isolated from 1 c.c. of the sprinkler effluent of Oct. 12th, 1905. For reasons which will appear later I have given it the name *B. hyponitrosus.* In form it is very short, almost coccus-like. It is actively motile and does not form spores. It does not retain the stain in Gram's method.

Gelatin plate culture: The colonies become visible after two days

at 22° C., as clear transparent round dew-drop like spheres. Under a low power of the microscope the *deep colonies* are perfectly round with a smooth contour and appear dark and granular. The *surface colonies* are larger and show a transparent wavy spreading margin, while the centre is dark and granular, the granulations becoming less marked towards the periphery. Usually a small dark round nucleus is seen of the same appearance as the deep colonies. This is often eccentrically situated and varies in size, apparently according to the depth in the gelatin from which the colony has grown to the surface. The surface colonies never exceed about 2 mm. in diameter. No liquefaction of the gelatin occurs.

Gelatin stroke culture: The growth is not unlike that of *B. coli*, but is rather harder and more adherent.

The growth on *agar* is soft, white, and not characteristic.

Milk is unchanged after 10 days at 37° C. No *indole* is formed in peptone water. In glucose broth growth does not readily take place. No growth takes place in ordinary broth under anaërobic conditions. If however nitrate be present, growth does occur. Even when exposed to air the growth in nitrate broth is more copious than that in ordinary broth.

In 0·25 % potassium nitrate broth the nitrate reaction disappears in from one to two days at 37° C., but no gas is produced.

Even in broth containing 1 % potassium nitrate growth takes place. In this strength of nitrate the growth at first takes the form of discrete colonies in the shape of small white spheres attached to the sides of the flask, or suspended freely in the liquid, the whole forming a very characteristic picture. Later diffuse growth occurs with uniform turbidity, and the nitrate reaction disappears in seven days at 37° C. Usually no gas is produced but I have occasionally observed some effervescence in cultures containing so much as 1 % nitrate.

This bacillus has now been grown on the ordinary laboratory media for a period of two and a half years since its original isolation. It grows readily at room temperature and does not require transplantation oftener than once a month. It has not changed its characters in any respect during this period. In particular its action on nitrates is as energetic as ever.

(4) *The Chemistry of B. hyponitrosus.*

As it appeared to be of considerable scientific interest, and of some practical importance with regard to the Belfast sewage problem, to

determine what becomes of the nitric nitrogen not appearing as gas, this question was fully investigated in connection with Bacillus No. 3. When this bacillus is grown in 1 % nitrate broth until the nitrate reaction has disappeared, the culture becomes strongly alkaline, and if a tube containing it be boiled, enough ammonia is given off to turn moist litmus paper blue. This at once suggested that the nitrate was reduced to ammonia, but as it was found that ammonia was also produced, although not in such large amount, in ordinary broth cultures of the bacillus, it became necessary to estimate accurately the amount of ammonia produced in each case.

Cultures were made in flasks containing 100 c.c. peptone water (peptone 10 gm., sodium chloride 5 gm. to 1 litre) to which varying quantities of potassium nitrate, accurately weighed, were added. The flasks after inoculation with the bacillus were incubated at 37° C., and any ammonia given off in the incubator was collected and estimated by connecting the flask with a test tube containing 10 c.c. deci-normal sulphuric acid. After incubation the culture was made up to 100 c.c. and 20 c.c. were taken for the estimation of ammonia. This was done by distillation with magnesia, *in vacuo*, at a temperature of from 35° to 40° C., according to the method described by Nencki and Zaleski (1895). The ammonia given off was received in a definite quantity of deci-normal sulphuric acid, which was afterwards titrated against deci-normal caustic soda, methyl orange being used as indicator.

In another sample of the culture the total alkalinity was estimated by titration against deci-normal sulphuric acid, with methyl orange as indicator; by deducting the amount of ammonia obtained by distillation, an estimate of the amount of "fixed alkali" present was obtained.

Estimations of the total unoxidised nitrogen by the Kjeldahl method were also made in some cases.

Exp. 1. This was a control experiment with sterile peptone water containing 0·25 % potassium nitrate.

20 c.c. on distillation with magnesia gave off ammonia requiring for its neutralisation 0·6 c.c. N/10 acid.

20 c.c. required to render it neutral to methyl orange 2·1 c.c. N/10 acid.

Therefore in 100 c.c.

NH_3 by distillation = 0·6 × 5 =	3·0 c.c. N/10 acid =	5·1 mgm.
Total alkali	10·5 c.c. N/10	„
Alkali not ammonia	7·5 c.c. N/10	„

Exp. 2. Culture in 100 c.c. peptone water, without nitrate, incubated for 48 hours at 37° C.

In 100 c.c.

NH_3 in incubator required	0·5 c.c. N/10 acid	
NH_3 by distillation = 1·4 × 5 =	7·0 c.c. N/10 „	
Total ammonia	7·5 c.c. N/10 „	= 12·75 mgm.

Exp. 3. Culture in 100 c.c. peptone water containing 0·254 gm. potassium nitrate; 48 hours at 37° C. A trace of nitrite was still present.

In 100 c.c.

NH_3 in incubator required	0·3 c.c. N/10 acid	
NH_3 by distillation = 2·7 × 5 =	13·5 c.c. N/10 „	
Total ammonia	13·8 c.c. N/10 „	= 23·46 mgm.
Total alkali	39·5 c.c. N/10 „	
Fixed alkali	26·0 c.c. N/10 „	

Exp. 4. Culture in 100 c.c. peptone water containing 0·56 gm. potassium nitrate; 48 hours at 37° C. Nitrate still present in considerable amount; a trace of nitrite.

In 100 c.c.

NH_3 in incubator required	0·3 c.c. N/10 acid	
NH_3 by distillation = 2·8 × 5 =	14·0 c.c. N/10 „	
Total ammonia	14·3 c.c. N/10 „	= 24·31 mgm.
Total alkali	36·5 c.c. N/10 „	
Fixed alkali	22·5 c.c. N/10 „	

In neither of the last two experiments had the nitrate reaction completely disappeared. They were therefore repeated, the cultures being allowed to remain in the incubator until the nitrate reaction was negative.

Exp. 5. Culture in 100 c.c. peptone water containing 0·25 gm. potassium nitrate; 3 days at 37° C. Nitrate reaction negative.

In 100 c.c.

NH_3 in incubator required	0·8 c.c. N/10 acid	
NH_3 by distillation = 3·2 × 5 =	16·0 c.c. N/10 „	
Total ammonia	16·8 c.c. N/10 „	= 28·56 mgm.
Total alkali	41·0 c.c. N/10 „	
Fixed alkali	25·0 c.c. N/10 „	

Exp. 6. Culture in 100 c.c. peptone water containing 0·50 gm. potassium nitrate; 5 days at 37° C. Nitrate reaction negative.

In 100 c.c.

NH_3 in incubator required	3·0 c.c. N/10 acid	
NH_3 by distillation = 5·3 × 5 =	26·5 c.c. N/10 „	
Total ammonia	29·5 c.c. N/10 „	= 50·15 mgm.
Total alkali	70·0 c.c. N/10 „	
Fixed alkali	43·5 c.c. N/10 „	

It is now desired to ascertain whether the increasing alkalinity of the culture exerted a retarding influence on the growth of the bacillus. This was done by adding to the culture at intervals, measured quantities of N acid in order to neutralise some of the alkali produced.

Exp. 7. Culture in 100 c.c. peptone water containing 0·52 gm. potassium nitrate; 5 days at 37° C. Nitrate reaction negative.

After 2 days 2·5 c.c. N sulphuric acid was added. On the next day the reaction was still strongly alkaline, and an additional 1·5 c.c. N acid was added. After 4 days nitrate was still present and the reaction was alkaline. In all 4·0 c.c. N acid was added, and 5 c.c. of the culture was withdrawn to test the alkalinity and the presence of nitrate. A correction for this had therefore to be made.

The total nitrogen was also estimated in this case by the Kjeldahl method.

In 100 c.c.

NH_3 in incubator required	0·3 c.c. N/10 acid	
NH_3 by distillation = 5·6 × 5 (20/19) =	29·5 c.c. N/10 „	
Total ammonia	29·8 c.c. N/10 „	= 50·6 mgm.
Total alkalinity	53·0 c.c. N/10 „	
Fixed alkali	23·5 c.c. N/10 „	
Add for 4 c.c. N acid	40·0 c.c. N/10 „	
Total fixed alkali	63·5 c.c. N/10 „	
Total nitrogen (Kjeldahl) as NH_3		= 170·5 mgm.

From the last two experiments we obtain the remarkable result that an alkalinity amounting to 70 % of a deci-normal solution (Exp. 6) does not interfere with the growth and vital activity of this bacillus. The amount of ammonia produced is practically identical, and the nitrate reaction disappeared in exactly the same time in both cases.

In the next experiment a still larger amount of nitrate was added; the culture was incubated until the nitrate reaction had disappeared, and the Kjeldahl nitrogen was again estimated.

Exp. 8. Culture in 100 c.c. peptone water containing 1·39 gm. potassium nitrate; 9 days at 37° C. Nitrate reaction negative.

In 100 c.c.

NH_3 in incubator required	2·0 c.c. N/10 acid	
NH_3 by distillation = 7·3 × 5 =	36·5 c.c. N/10 „	
Total ammonia	38·5 c.c. N/10 „	= 65·45 mgm.
Total alkali	114·0 c.c. N/10 „	
Fixed alkali	78·0 c.c. N/10 „	
Total nitrogen (Kjeldahl) as NH_3		= 163·2 mgm.

Here the alkalinity of the culture is greater than that of a decinormal solution. The amount of ammonia is only slightly greater than in the previous experiment, and the Kjeldahl nitrogen, which of course includes the ammonia, is rather less.

Exp. 9. An estimation of the total nitrogen in sterile 1 % peptone water was now made by the Kjeldahl method.

After incineration and distillation the ammonia given off from 10 c.c. required for its neutralisation

in the first sample	9·9 c.c. N/10 acid
in the second sample	10·3 c.c. „
Average	10·1 c.c. „

In 100 c.c. therefore

Total nitrogen (Kjeldahl) as NH_3 = 171·7 mgm.

This is rather greater than the amounts obtained in Exps. (7) and (8) where amounts of nitrate equivalent respectively to 88 mgm. and 236 mgm. of ammonia had disappeared.

These results show clearly that the ammonia produced in the cultures is derived, not from the nitrate, but from the peptone.

We may now tabulate in a different form some of the results already obtained.

Deducting the amount of ammonia (5·1 mgm.) in the sterile peptone water from that in the cultures, we find the following figures for the amounts of ammonia produced.

Exp. No.	Days	Nitrate	NH_3 produced
(2)	2	nil	7·65 mgm.
(3)	2	0·25 %	18·36
(4)	2	0·56	19·21
(5)	3	0·25	23·46
(6)	5	0·50	45·05
(7)	5	0·52	45·50
(8)	9	1·39	60·35

The presence of nitrate undoubtedly causes an increase in the formation of ammonia. This must be accounted for by the fact that it favours the growth of the bacillus enabling it to break down the peptone more rapidly. The amount of ammonia produced is however much more nearly proportionate to the age of the culture than to the amount of nitrate present.

If similarly we deduct the amount of "fixed alkali" found in the control experiment (7·5 c.c. N/10) from that found in the cultures, and suppose for the present that the remainder is due to KOH derived from KNO_3, it is easy to calculate what percentage of the nitrate present is represented by the fixed alkali produced.

Exp. No.	Fixed alkali	KNO_3	Percentage of total KNO_3
(3)	18·5 c.c. N/10	187 mgm.	73·6 %
(4)	15·0 c.c.	152	25·0
(5)	17·5 c.c.	177	71·0
(6)	36·0 c.c.	364	72·9
(7)	56·0 c.c.	565	108
(8)	70·5 c.c.	713	51·1

We have seen that the nitrate disappearing is not represented by ammonia, nor by any other form of nitrogen which appears in a Kjeldahl analysis. If it disappeared in gaseous form, either as free nitrogen or as lower oxides, the whole of the potassium nitrate disappearing would necessarily be represented by fixed alkali in the culture. That this is not so, except in one case (Exp. 7), is clear from the above table. The fixed alkali accounts only for, at most, 73 % of the potassium nitrate.

This suggests, as the true explanation of the facts, that the nitrate is reduced to *hyponitrite.*

Divers (1899) has shown that when potassium amalgam is made to act on potassium nitrate, among other products of reduction, potassium hyponitrite (KNO) is formed. The hyponitrites of sodium and potassium are, like the carbonates, salts with an alkaline reaction. They are rather unstable, and on heating, with or without acid, they give off nitrous oxide gas (N_2O). They do not give the brucin sulphuric acid reaction, and they do not appear in a Kjeldahl analysis as they are at once decomposed on heating with sulphuric acid.

The "fixed alkali" therefore, in the above experiments, represents not a portion of the KNO_3 as KOH, but the whole of it as KNO. In the exceptional experiment (No. 7), sufficient sulphuric acid had been added to decompose most of the hyponitrite with formation of K_2SO_4,

and the alkali in combination with sulphuric acid is reckoned in the total fixed alkali produced.

That bacillus No. 3 reduces nitrate to hyponitrite and no further, and may therefore appropriately be named *B. hyponitrosus*, is further shown by analyses of the gas given off from cultures in nitrate peptone water when these are boiled with acid. It is possible to recover in this way the whole of the nitrogen disappearing as nitrate, in the form of nitrous oxide gas.

The gas analyses were carried out by means of the apparatus described by Haldane (1898), in which there is an automatic adjustment for changes of temperature and pressure during a series of observations.

Exp. 10. A large flask of 0·5 % nitrate peptone water was inoculated with the bacillus and incubated at 37° C. until the nitrate reaction had disappeared. Sulphuric acid was then added, the flask gently heated over a Bunsen burner, and the gas given off collected over mercury. The first samples tested contained a considerable amount of carbonic acid as was shown by shaking up with lime water. Another sample of the gas collected in a test tube, after absorption of carbonic acid by means of moistened solid caustic soda, caused a burning splinter of wood to glow more brightly. This is evidence of the presence of nitrous oxide, as oxygen is practically out of the question. A small quantity of a strong solution of caustic soda was now introduced into a burette over mercury, the flask was heated gradually almost to boiling point, and the gas not absorbed by the caustic soda was collected and analysed.

A sample introduced into Haldane's apparatus measured 7·15 c.c. An approximately equal volume of hydrogen from a Kipp apparatus was introduced, the reading being now 14·66 c.c. The mixture was now ignited in an explosion pipette; an explosion took place, accompanied by a reduction in volume to 8·65 c.c. The amount of hydrogen used was therefore 6·01 c.c. and this is equivalent to the amount of nitrous oxide in the sample of 7·15 c.c., that is to say, 84 % of the whole.

Another sample of the gas was introduced and passed over into the pyrogallic bulb. Some absorption took place. The remainder formed an explosive mixture with hydrogen as before and contained 64·5 % of nitrous oxide. By this means the possibility of the explosion being due to oxygen is excluded, but on account of the great solubility of nitrous oxide it is impossible to obtain an accurate analysis if the gas is subjected to absorption before explosion.

In the next experiment the amount of nitrous oxide obtained from a definite quantity of nitrate was measured, and the amount of nitrous oxide given off on boiling the culture without the addition of acid was also ascertained. In each case the gas was boiled off under slightly reduced pressure by means of a special apparatus consisting of a large pipette connected to a mercury reservoir and surrounded by a steam jacket. From this apparatus it was easy to collect the gas in a nitrometer over mercury without any possibility of contamination with air. The gas collected was measured in the nitrometer, the temperature and barometric pressure being noted. Samples were then transferred to Haldane's apparatus for analysis. Enough residual nitrogen was left in the gas-analysis apparatus to prevent explosion and the combination with hydrogen was effected by means of the combustion pipette. This contains a spiral of platinum wire which can be brought to a bright red heat by means of a storage battery. After combustion the carbonic acid was estimated by absorption in the potash bulb.

Exp. 11. A flask of peptone water to which exactly 0·50 gm. potassium nitrate had been added was inoculated with *B. hyponitrosus* and incubated at 37° C. for 3 days. The nitrate reaction was then negative.

The whole culture measured exactly 225 c.c. Of this definite fractions were taken for analysis as follows.

From 60 c.c. of the culture, on boiling with acid, there was obtained

21 c.c. of gas at 21° C. and 758 mm.

The analysis of this gas gave:

	First sample	Second sample	Average
Nitrous oxide	90·7 %	91·4 %	91·0 %
Carbonic acid	6·5	5·5	6·0
Nitrogen	2·8	3·1	3·0

Thus from $60/225 \times 0{\cdot}5$ gm. KNO_3 we obtain

$21 \times 91/100$ c.c. N_2O at 21° C. and 758 mm.,

therefore 1 gm. KNO_3 would yield

143·3 c.c. N_2O at 21° C. and 758 mm.

or (correcting for temperature and pressure)

133 c.c. N_2O at 0° C. and 760 mm.,

and from 1 gm. KNO_3, 135 c.c. N_2O „ „

is the theoretical amount.

As a control the gas from another sample of the culture was analysed by absorption in the potash and pyrogallic bulbs without combustion. This gave the following:

Nitrous oxide + carbonic acid	98 %
Nitrogen	2 %

This again excludes the presence of oxygen. The nitrous oxide is not so readily absorbed by the potash as is carbonic acid, but by continuing the absorption until a constant reading was obtained, 98 % of the whole sample disappeared, and no further absorption occurred in the pyrogallic bulb.

From 35 c.c. of the culture, on boiling without acid, there was obtained

7 c.c. of gas at 20° C. and 762 mm.

The analysis of this gas gave:

Nitrous oxide	81·5 %
Carbonic acid	7·6
Nitrogen	10·9

Thus from $35/225 \times 0{\cdot}5$ gm. KNO_3 we obtain

$7 \times 81{\cdot}5/100$ c.c. N_2O at 20° C. and 762 mm.

therefore 1 gm. KNO_3 would yield

73·4 c.c. N_2O at 20° C. and 762 mm.

or 68 c.c. N_2O at 0° C. and 760 mm.

Thus we obtain as nitrous oxide the following percentages of the total nitrate present,

on boiling the culture with acid	98·5 %
on boiling the culture without acid	50·3 %

This difference shows that the nitrous oxide is not present merely in solution, but that it is in combination as hyponitrite, which is decomposed more easily in acid than in alkaline solution on boiling.

Thus we find that in cultures of *B. hyponitrosus* in nitrate peptone water, the whole of the nitrate is reduced to hyponitrite, and remains in solution in this form, at least where the total amount of nitrate originally present does not exceed 0·25 %.

As has already been mentioned, in some cases gas formation does occur in cultures containing 1 % potassium nitrate. This is not a constant occurrence, and it was not found possible to collect the gas in sufficient quantities for a satisfactory analysis, but on one occasion I was able to detect in it the presence of nitrous oxide. The reason for

the difficulty in obtaining a sufficient quantity of the gas for analysis appears to be that the strictly anaërobic conditions necessary for the collection of the gas are not favourable to the growth of the bacillus. Under these conditions, in 1 % nitrate peptone water, the growth only proceeds to the stage of discrete colonies; no diffuse growth occurs and only a small amount of gas is given off.

(5) *The relation of Oxygen to the growth of the Denitrifying Bacteria.*

The denitrifying bacteria are essentially aërobic, they can however grow under anaërobic conditions in the presence of nitrate, the nitrate supplying the necessary oxygen for their vital activities. On the other hand, under conditions of specially good aëration, these bacteria grow readily enough but they do not attack nitrate, the necessary oxygen being otherwise supplied.

Experiments on these points were made both with pure cultures of Bacilli Nos. 1, 2 and 3, and with sewage samples.

Cultures of the three bacilli were made in test tubes containing 0·25 % nitrate broth and in the same broth without the addition of nitrate. One set of the cultures were placed under anaërobic conditions in Buchner's tubes. After incubation at 37° C. the anaërobic cultures in ordinary broth showed no growth. The order of the amount of growth in the other cases was as follows:

(1) Aërobic nitrate, (2) Aërobic, (3) Anaërobic nitrate.

In the nitrate cultures denitrification proceeded at the same rate under aërobic and anaërobic conditions.

The next experiments were done with a view to determine whether denitrification takes place under conditions of specially good aëration.

(1) Cultures were made in dilute peptone broth (1 in 20) containing 0·03 % potassium nitrate, in small Erlenmeyer flasks, each flask receiving only 10 c.c. of the broth so that the liquid was spread out in a thin layer on the bottom of the flask and freely exposed to air.

In the case of Bacilli Nos. 1 and 2, the nitrate reaction was still present after 3 days at 37° C. In the case of Bacillus No. 3, the nitrate reaction had disappeared in 3 days; it was not tested earlier.

In ordinary test tube cultures containing 10 c.c. of this dilute nitrate broth the nitrate reaction in the case of all three bacilli disappears in 2 days at 37° C.

(2) Cultures of Bacillus No. 3 were made in 0·25 % potassium

nitrate broth, (*a*) in test tubes containing 10 c.c., and (*b*) in Erlenmeyer flasks containing 10 c.c.

After 2 days at 37° C. the nitrate reaction had disappeared in the test tube, but was still present in the flask.

(3) Cultures of Bacillus No. 2 were made in 0·25 % potassium nitrate broth, (*a*) in test tubes containing 10 c.c., and (*b*) in Erlenmeyer flasks containing 10 c.c.

The cultures were incubated for 3 days at 37° C. and then allowed to stand for 2 days at room temperature.

Each culture was then made up to 50 c.c. with water, and the nitrite present was estimated by Griess' method. In another sample the nitrite was got rid of by heating in acid solution with urea, and the absence of nitrate in both cases demonstrated by the brucin method.

The percentage of the original nitrate remaining as nitrite was

(*a*) in test tube 9·3 %,

(*b*) in flask 33·9 %.

(4) Samples of septic tank and sprinkler effluents were mixed in equal quantities and allowed to stand in a stoppered bottle three quarters full. After 24 hours at room temperature the nitrate reaction had disappeared.

(5) 30 c.c. each of the same septic tank and sprinkler effluents were put in a large Erlenmeyer flask so as to form a layer about 1/8 inch in depth and expose a large surface in contact with air. After 4 days at room temperature the nitrate reaction was still present.

These results show that denitrification does not proceed so rapidly either in pure cultures of the bacteria, or in the sewage, when there is free exposure to air.

(6) *Estimation of the number of Denitrifying Bacteria in the various samples of Sewage.*

Dilutions of the sewage samples were made as before, and two distinct methods were used for demonstrating the presence of denitrifying bacteria.

First method. 1 c.c. of each dilution tested was added to a tube filled to 10 c.c. with dilute nitrate peptone water (0·02 % potassium nitrate); the tubes were incubated for 3 to 4 days and then tested for the nitrate reaction. The disappearance of this reaction was taken as evidence of the presence of denitrifying bacteria.

Second method. Durham's fermentation tubes containing 0·25 %

nitrate broth were used, and the appearance of well marked gas formation (at least 1/10 of the closed limb) was regarded as evidence of the presence of typical denitrifying bacteria belonging to the class represented by *B. Stutzeri.*

In using nitrate containing media it was kept in mind that there is a class of bacteria represented by *B. denitrificans*, which decompose nitrite with formation of free nitrogen, but which do not attack nitrate. As however many of the bacteria present in sewage can reduce nitrate to nitrite, it seemed better to use the nitrate media, and to interpret the results as indicating the presence of either (1) bacteria capable of destroying nitrate, or (2) a combination of bacteria which can reduce nitrate to nitrite with nitrate destroying organisms.

On Dec. 12th, the samples were examined by both the methods mentioned above, and the detailed observations for that date are given below as an example of the results obtained.

	Dilutions				
Sample	1/10	1/100	1/1000	1/10,000	1/100,000
First method:					
S. T. effluent	–	+	+	+	no growth.
Sp. effluent	+	+	0	0	,,
Upper Denitrifying	–	+	+	0	,,
Lower Denitrifying	–	+	+	0	,,
Second method (gas formation):					
S. T. effluent	–	+	+	0	0
Sp. effluent	0	0	0	no growth	
Upper Denitrifying	–	+	0	0	0
Lower Denitrifying	–	0	0	0	no growth.

In the above table

\+ indicates disappearance of nitrate reaction, or gas formation.

0 indicates positive nitrate reaction, or no gas formation, although growth occurred.

– indicates "not tested."

In these samples denitrifying bacteria which do not produce gas are obviously more numerous than the typical denitrifiers. Thus with 1/10 c.c. sprinkler effluent, and 1/100 c.c. effluent from the lower "denitrifying" bed, no gas formation occurred although the nitrate reaction disappeared with 1/100 and 1/1000 c.c. respectively. In this case larger quantities were not tested, but it is to be noted that if a sufficient quantity of any of the sewage samples were inoculated into 0·25 % nitrate broth, gas formation always occurred. This suggests

that under natural conditions all the nitrate disappears as free nitrogen. We know that the formation of nitrite is a constant stage in the reduction of nitrate both by the typical denitrifiers and by those belonging to the *B. hyponitrosus* group. It is probable that in a similar way hyponitrite also represents a stage in the reduction of nitrate by the typical denitrifiers, and that in the presence of both classes of bacteria, the reduction proceeds further, with formation of free nitrogen. This is a point which requires further investigation.

For comparison with the numbers of denitrifying bacteria, enumerations were also made of *B. coli* and streptococci, and also of the total bacteria growing at 22° and 37° C.

The results of the enumerations on the various dates are given in Table VI, and also the averages for the whole series. It will be observed that the figures agree very closely with the counts made in February (Table III).

A very large reduction of bacteria in the sprinkler effluent is again seen and this is more marked in the denitrifiers than in any other group. *B. coli* is about four times as numerous as the denitrifying bacteria, and of these, as we have already seen, the class which does not produce gas is the more frequent. The great reduction in the denitrifying bacteria in the sprinkler effluent is further evidence that denitrification does not readily occur where there is very free aëration.

But the fact that denitrifying organisms do occur in considerable numbers in the sprinkler effluent calls for remark. When the sprinkler effluent is incubated it does not lose its nitrate (Exp. 2, p. 625) although denitrifying bacteria are present in it. This can only be because it does not contain enough organic material to serve for their growth. It was in fact found that Bacillus No. 2 could not grow in sprinkler effluent which had been filtered through porcelain. If however a small amount of nutrient broth were added, growth and denitrification took place.

(7) *Conclusions.*

We have seen in the foregoing that the combination of a percolating bed (the sprinkler), with contact beds in which denitrification occurs, gives rise to a final effluent very similar both in chemical and bacteriological characters to that obtained by double contact treatment.

It is probable therefore that in the contact beds a considerable proportion of the organic nitrogen disappears as gas as a result of alternate nitrification and denitrification. In the sprinkler the free exposure to air renders it unlikely that any appreciable amount of denitrification

occurs. We may therefore take the excess of nitrate in the sprinkler effluent over that present in the effluent from the contact beds as a rough indication of the amount of nitrogen disappearing as gas in double contact treatment. This amounts to about 50 % of the nitrogen disappearing from the contact bed effluent (Table IV).

We can now easily understand why a contact bed effluent as a rule shows less nitrate than the effluent from a percolating bed. The amount of nitrate present is a direct measure of the degree of aëration to which the sewage has been exposed. Equally good effluents as regards purification may be obtained by means of contact treatment, or from a continuously working percolating bed. In the former a large amount of nitrogen disappears as gas; in the latter it is represented by nitrate. If the nitrate disappears completely from a contact bed effluent it is of course a sign that the bed is being overworked, the denitrifying process has been allowed to proceed too far; in other words the bed has been filled too frequently or too long, and sufficient interval has not been allowed for aëration and the active processes which accompany it during the so-called "resting" stage.

It also becomes clear why a highly nitrated effluent is less liable to undergo objectionable putrefactive changes than one less rich in nitrates, but otherwise similar. The denitrifying bacteria utilise the oxygen of the nitrate in breaking down complex nitrogenous substances under what are practically aërobic conditions. Anaërobic putrefaction with its resulting objectionable products is thus, to some extent at least, prevented. It is only putting the matter in another way to say that the nitrate oxidises the organic matter present. That it does so is due to the activity of the denitrifying bacteria. When *B. hyponitrosus* is grown in peptone solution containing nitrate it breaks down the peptone into ammonia much more readily than in the absence of nitrate. In neither case does it produce any indole, a typical product of putrefaction. So long as any nitrate is present, the cultures of this bacillus have no putrefactive odour. When the nitrate disappears there is a slight, but not very offensive odour of putrefaction.

The practical result of this study of denitrification, in its application to the Belfast sewage problem, appears to be that it is impossible to get rid of all the nitrate in the effluent without materially lessening the purification effected. The amount of organic material in a good effluent like that from the sprinkler is not sufficient for the growth of the denitrifying bacteria, and as we have seen, the disappearance of nitrate from an effluent is a sign that the bed is being overworked.

It may indeed, with reason, be asked whether it is desirable, even under the special conditions obtaining in Belfast, to try to get rid of any of the nitrate.

It is not certain that the living green sea-weeds can assimilate any appreciable amount of nitrate; and it is not improbable, on the other hand, that the nitrate may be very valuable in preventing objectionable putrefactive changes in the dead sea-weed, in virtue of the activity of the denitrifying bacteria.

NITRIFICATION AND OTHER BACTERIAL PROCESSES.

That the rôle of bacteria in the purification of sewage is a somewhat limited one, which by no means justifies the application of such terms as "bacteria beds" and "bacterial purification," appears from what has already been said. There is however one other stage in the process which may with great probability be ascribed to bacterial action, the stage namely of nitrification.

The nitrifying bacteria, first isolated and studied by Winogradsky, are divided into two groups, viz.

(1) *Nitrite bacteria* which convert ammonia into nitrite.

(2) *Nitrate bacteria* which carry the oxidation a stage further, and convert nitrite into nitrate.

These organisms do not grow on the ordinary laboratory media used for the cultivation of other bacteria. Their growth, in pure culture at least, is inhibited by the presence of more than minimal amounts of organic material, the nitrite formers being in this respect more sensitive than the nitrate formers. On the other hand, the presence of ammonia in any appreciable quantity inhibits the growth of the nitrate bacteria. Hence, when ammoniacal solutions suitable for the growth of the nitrite bacteria, are inoculated with a mixture of the two kinds, the process of nitrification takes place in two distinct stages, no nitrate being formed until practically all the ammonia has been converted into nitrite.

In view of these facts it is difficult to correlate the nitrification which occurs in nature, and in particular that which occurs in sewage purification, with the activity of these bacteria. In nature the process proceeds rapidly to the stage of nitrate in the presence of a large amount of organic material, and in spite of the presence of a large amount of ammonia.

A reasonable explanation of this difficulty has however been given by Winogradsky (1904).

This depends in the first place upon observations made by Omelianski (1899), who has shown that in combination with *B. ramosus*, a common soil bacterium of the "subtilis" group, the nitrite former can grow and cause the oxidation of ammonia, even in dilute peptone broth. When, under certain conditions, *B. ramosus* and the nitrite former are grown together in dilute peptone broth (1 in 20) ammonia is first produced and after 7 days nitrite appears. When in addition to these two bacteria the nitrate former is inoculated in the dilute broth, nitrate is formed, but only after about a month. It appears therefore that under conditions such as occur in nature the growth and activity of the nitrite forming bacteria are not interfered with by the presence of organic material.

The further difficulty of the formation of nitrate in the presence of ammonia is explained by the results of certain experiments of Boullanger and Massol (1903). These observers showed that when an ammoniacal solution, inoculated with both forms of the nitrifying bacteria, was allowed to undergo complete oxidation in contact with clinker and then withdrawn, a further supply of the ammoniacal solution poured on to the same clinker did not then interfere with the activity of the nitrate formers, but the oxidation proceeded at once as far as nitrate and only traces of nitrite could be found. In this case a growth of the nitrate forming bacteria had been established on the surface of the clinker, in a manner which exactly corresponds to what takes place in the "ripening" of a bed in sewage purification; and it appears that ammonia has a much greater power of inhibiting the growth of the nitrate forming bacteria than of interfering with their activity when growth has been once established.

In view of these two sets of observations, the conditions of which correspond to those present in the treatment of sewage in a percolating bed, there is good reason for ascribing the nitrification which occurs there to the activity of the nitrifying bacteria described by Winogradsky.

Before we can rightly estimate the relative importance of bacteria and of the higher forms of life in the chemical changes which lead to the regeneration of the adsorptive capacity of a bed much more work requires to be done. In particular the fauna and flora of an efficiently working bed should be studied and contrasted with those of a bed which is being overworked or is in the condition known to engineers as "sick."

There can be little doubt that bacteria play an important part in breaking down the large proteid molecule of those colloid substances

which are readily "adsorbed"; the simpler substances thus produced are easily oxidised to soluble inorganic compounds which are no longer retained by adsorption but pass out into the effluent. How far this oxidation is a purely chemical process and how far it is dependent on the activity of vegetable and animal forms of life, is a matter which requires further investigation. In the case of ammonia the oxidation to nitrate is, as we have seen, almost certainly dependent on bacterial action. On the other hand the great destruction of bacteria which takes place in the beds can be most readily accounted for by the activity of higher forms of life. It is however possible that the presence of adsorbed or condensed oxygen, while it favours the growth of the nitrifying micro-organisms, is unfavourable to the existence of the ordinary bacteria.

The work described in this paper was carried out partly in the Pathological Laboratory of Queen's College, Belfast, and partly in the Pathological Laboratory of the University of Manchester. Part of the work on denitrification was done jointly with Dr T. Carnwath.

I desire here to express my thanks to Prof. Letts and Prof. Milroy for their kind assistance in the chemical part of the work, and to Prof. Lorrain Smith and Dr C. Powell White for valuable suggestions at various stages of the investigation.

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TABLE I.

Detailed Results of First Series.

Crude Sewage.

Date	Bacteria per c.c. Gelatin at 22° C.	Agar at 37° C.	Spores	*B. coli* present in fraction of 1 c.c.	*Streptococci* (or number per c.c.)	*B. enteritidis*
July 22, '04	16,000,000	—	7,500	1/100,000	—	1/1000
,, 26	90,000,000	—	500	(160,000)	—	1/100
Aug. 5	9,000,000	4,500,000	470	1/100,000	1/100,000	1/100
,, 12	40,000,000	3,300,000	1,700	1/100,000	(less than 100,000)	(less than 10)
,, 23	16,300,000	1,500,000	490	1/100,000	1/1000,000	1 c.c.
Average	34,000,000	3,100,000	2,130			

Screened and Settled.

July 22	17,000,000	—	5,500	1/100,000	1/100,000	1/1000
,, 26	20,000,000	—	700	(220,000)	—	1/100
Aug. 5	11,000,000	2,300,000	220	1/100,000	1/100,000	1/1000
,, 12	23,000,000	2,300,000	530	1/1000,000	(less than 100,000)	(less than 10)
,, 23	15,000,000	700,000	240	1/100,000	1/1000,000	1 c.c.
Average	17,200,000	1,760,000	1,440			

Septic Tank.

July 22	10,000,000	—	(less than 1,000)	1/100,000	—	1/100
,, 26	4,000,000	—	530	(60,000)	—	1/100
Aug. 5	6,000,000	1,200,000	160	1/1000,000	(less than 100,000)	1/100)
,, 12	10,000,000	450,000	690	1/100,000	(less than 100,000)	(less than 10)
,, 23	12,000,000	560,000	320	1/1000,000	1/100,000	1 c.c.
Average	8,400,000	736,000	540			

Lower A. Bed Effluent.

July 22	1,000,000	—	(less than 100)	(less than 100,000)	—	1/100
,, 26	1,700,000	—	—	(20,000)	—	1/100
Aug. 5	6,500,000	400,000	40	1/100,000	1/100,000	1/10
,, 12	1,400,000	(less than 100,000)	140	1/100,000	(less than 100,000)	1/100
,, 23	2,700,000	320,000	90	1/100,000	1/10,000	—
Average	2,660,000	270,000	90			

Lower B. Bed Effluent.

July 22	5,000,000	—	(less than 100)	1/100,000	—	1/100
,, 26	1,700,000	—	800	(45,000)	—	1/100
Aug. 5	4,500,000	1,100,000	150	1/100,000	1/100,000	1/10
,, 12	2,500,000	600,000	250	1/1000,000	(less than 100,000)	(less than 10)
,, 23	3,900,000	470,000	150	1/100,000	1/1000,000	—
Average	3,780,000	723,000	290			

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TABLE I. (*Continued.*)

Lower D. Bed Effluent.

Date	Bacteria per c.c. Gelatin at 22° C.	Agar at 37° C.	Spores	*B. coli* present in fraction of 1 c.c.	*Streptococci* (or number per c.c.)	*B. enteritidis*
July 22	1,000,000	—	—	(less than 100,000)	—	1/100
,, 26	3,000,000	—	500	1/100,000	—	1/10
Aug. 5	3,000,000	500,000	100	1/100,000	(less than 100,000)	1/100
,, 12	2,100,000	400,000	400	1/100,000	(less than 100,000)	(less than 10)
,, 23	5,200,000	750,000	150	1/1000,000	1/100,000	—
Average	2,850,000	550,000	290			

Lower F. Bed Effluent.

July 22	1,500,000	—	(2,100?)	—	—	1/100
,, 26	2,000,000	—	200	1/100,000	—	1/10
Aug. 5	3,000,000	900,000	210	(less than 100,000)	(less than 100,000)	1/10
,, 12	3,150,000	500,000	370	1/100,000	(less than 100,000)	(less than 10)
,, 23	3,800,000	470,000	170	1/1000,000	1/10,000	1 c.c.
Average	2,690,000	623,000	230			

Lower G. Bed Effluent.

July 22	1,000,000	—	—	1/100,000	—	1/20
,, 26	2,700,000	—	300	(50,000)	—	1/100
Aug. 5	5,000,000	850,000	90	(less than 100,000)	1/100,000	1/100
,, 12	2,500,000	350,000	290	(less than 100,000)	(less than 100,000)	(less than 10)
,, 23	3,300,000	410,000	130	1/100,000	1/10,000	1 c.c.
Average	2,900,000	536,000	200			

(Note:—The numbers given in brackets for *B. coli* on July 26th were obtained from counts on Drigalski-Conradi plates.)

Untreated Sewage.

(i.e. Crude Sewage, Screened and Settled, and Septic Tank).

	B. coli 15 examinations	*Streptococci* 10 examinations	*B. enteritidis* 15 examinations
In 1/10,000 c.c.	1 time	4 times	6 times in 1 c.c.
In 1/100,000 c.c.	11 times	4 ,,	6 times in 1/100 c.c.
In 1/1000,000 c.c.	3 ,,	2 ,,	3 times in 1/1000 c.c.
Average	886,000 per c.c.	813,000 per c.c.	800 per c.c.

Effluents from Contact Beds.

	B. coli 24 examinations	*Streptococci* 15 examinations	*B. enteritidis* 22 examinations
In 1/10,000 c.c.	8 times	10 times	6 times in 1 c.c.
In 1/100,000 c.c.	13 ,,	4 ,,	6 times in 1/10 c.c.
In 1/1000,000 c.c.	3 ,,	1 time	10 times in 1/100 c.c.
Average	607,000 per c.c.	332,000 per c.c.	161 per c.c.

Per cent. Reduction in Numbers in the Effluents.

B. coli	*Streptococci*	*B. enteritidis*
31·5 %	59·2 %	80 %

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TABLE II.

Average of Five Examinations, First Series.

	Bacteria per c.c.		
	On gelatin at 22° C.	On agar at 37° C.	Spores
Crude Sewage	34,260,000	3,100,000	2130
Screened and settled	17,200,000	1,760,000	1440
Septic Tank	8,400,000	736,000	540
Bed A	2,660,000	273,000	90
Bed B	3,780,000	723,000	290
Bed D	2,850,000	550,000	290
Bed F	2,690,000	623,000	230
Bed G	2,900,000	536,000	200
(Aug. 23, Sprinkler	1,400,000	80,000	100)

	Per cent. reduction on crude sewage. Bacteria			Per cent. reduction on crude sewage. Chemical results			Per cent. reduction on septic tank. Bacteria		
	Gelatin	Agar	Spores	Free NH_3	Alb. NH_3	Oxyg. abs. test	Gelatin	Agar	Spores
Screened Sewage	50·0	43·0	31·0	(gain) 12	25	23	—	—	—
Septic Tank	75·4	76·3	74·0	(gain) 40	45	38	—	—	—
Bed A	92·3	91·2	95·8	(loss) 85	74	83	68·3	63	83
Bed B	89·0	76·7	86·0	43	71	77	55·0	2	46
Bed D	92·0	82·3	86·0	56	73	81	66·0	26	46
Bed F	92·2	80·0	89·0	52	79	79	68·0	16	57
Bed G	91·6	83·0	90·0	37	73	73	65·5	28	63
(Aug. 23 Sprinkler	91·5	94·7	—	—	—	—	88·4	86	—)

(Note :—The bacteria growing on agar at 37° C. were only counted on three occasions, and the figures for the sprinkler are based on one examination.)

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TABLE III.

Detailed Results of Second Series.

Date	Bacteria per c.c. Gelatin at 22° C.	Bacteria per c.c. Agar at 37° C.	*B. coli* present in fraction of 1 c.c.	*Streptococci* present in fraction of 1 c.c.
		Septic Tank.		
Jan. 25, '05	3,750,000	220,000	1/100,000	(1/1000,000)
Feb. 1	2,700,000	100,000	1/10,000	1/10,000
,, 8	4,500,000	450,000	1/100,000	1/10,000
,, 15	6,150,000	1,500,000	1/100,000	1/100,000
,, 22	350,000	210,000	1/10,000	1/1000
Mar. 1	2,300,000	170,000	1/100,000	1/10,000
Average	3,300,000	440,000	349,000	133,000 per c.c.
		Sprinkler Effluent.		
Jan. 25	500,000	35,000	1/10,000	1/10,000
Feb. 1	250,000	5,000	(less than 10,000)	(less than 10,000 per c.c.)
,, 8	180,000	6,000	1/1000	1/10,000
,, 15	285,000	57,000	1/1000	1/1000
,, 22	5,000	3,000	(less than 1000)	(less than 1000 per c.c.)
Mar. 1	110,000	2,500	1/1000	(less than 1000 per c.c.)
Average	220,000	18,000	8,000	12,000 per c.c.
		Mixture of Septic Tank and Sprinkler Effluent (calculated).		
Jan. 25	2,100,000	127,000	—	—
Feb. 1	1,500,000	52,000	—	—
,, 8	2,300,000	230,000	—	—
,, 15	3,200,000	780,000	—	—
,, 22	200,000	120,000	—	—
Mar. 1	1,400,000	120,000	—	—
Average	1,780,000	238,000	178,000	72,000 per c.c.
		Effluent from 1st Contact Bed (Upper denitrifying).		
Jan. 25	950,000	45,000	1/10,000	1/10,000
Feb. 1	1,200,000	50,000	1/10,000	(less than 10,000 per c.c.)
,, 8	3,000,000	150,000	1/10,000	1/100,000
,, 15	2,900,000	890,000	1/10,000	1/10,000
,, 22	150,000	40,000	1/1000	1/10,000
Mar. 1	1,800,000	51,000	1/10,000	1/10,000
Average	1,670,000	204,000	42,000	79,000 per c.c.
		Effluent from 2nd Contact Bed (Lower denitrifying).		
Feb. 22	125,000	30,000	1/1000	1/1000
Mar. 1	600,000	19,000	1/1000	1/1000
Average	360,000	25,000	(less than) 10,000	(less than) 10,000 per c.c.

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TABLE IV.

Detailed Results of Second Series.

Chemical Examinations by Prof. Letts.

Septic Tank.

Date	Oxygen absorbed (4 hours at 27° C.)	Parts per 100,000 — Nitrogen as: Free NH_3	Albuminoid NH_3	Nitrates	Nitrites
Jan. 25, '05	7·98	3·46	0·70	—	—
Feb. 1	6·24	3·30	0·74	—	—
,, 8	6·88	2·96	0·74	—	—
,, 15	6·88	3·62	0·82	—	—
,, 22	5·95	2·58	0·62	—	—
Mar. 1	5·33	3·29	0·47	—	—
Average	6·54	3·20	0·68	—	—

Sprinkler Effluent.

Jan. 25	2·68	1·48	0·39	1·32	—
Feb. 1	2·59	1·07	0·33	1·32	traces
,, 8	2·56	0·74	0·37	1·51	,,
,, 15	2·56	1·43	0·41	1·40	0·12
,, 22	2·27	0·49	0·29	1·98	traces
Mar. 1	2·07	0·56	0·27	2·46	0·01
Average	2·45	0·96	0·33	1·66	—

Effluent from 1st Contact Bed (Upper denitrifying).

Jan. 25	3·05	1·48	0·37	(0·82)*	0·0
Feb. 1	2·96	1·40	0·37	0·00	0·0
,, 8	3·04	1·32	0·37	0·00	0·0
,, 15	3·04	1·48	0·45	0·00	0·0
,, 22	2·49	0·95	0·37	0·00	0·0
Mar. 1	2·27	0·93	0·27	0·25	0·0
Average	2·80	1·26	0·36	0·05	—

Effluent from 2nd Contact Bed (Lower denitrifying).

Feb. 22	1·95	0·58	0·25	0·12	traces
Mar. 1	1·74	0·82	0·21	0·33	—
Average	1·84	0·69	0·23	0·26	—

* The upper denitrifying bed had only recently been started; this figure is therefore not included for the average.

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TABLE V.

Average of Six Examinations, Second Series.

			Sprinkler	Denitrifying Beds: Upper	Denitrifying Beds: Lower
Percentage reduction in bacteria calculated on...	Septic Tank ...	Gelatin at 22° C.	93·4 %	50 %	72 %
		Agar at 37° C.	96·0	53	87
	Mixture of Septic Tank & Sprinkler	Gelatin at 22° C.	—	6	53
		Agar at 37° C.	—	14	77
Percentage chemical purification calculated on...	Septic Tank ...	Oxygen absorbed	63	57	67
		Free NH_3	71·5	61	76
		Alb. NH_3	51	48	55
	Mixture of Septic Tank & Sprinkler	Oxygen absorbed	—	37	56
		Free NH_3	—	39	63
		Alb. NH_3	—	28	44

(Note :—There were only two examinations of the effluent from the lower denitrifying bed.)

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TABLE VI.

Enumeration of Denitrifying Bacteria, etc.

Septic Tank.

(Disappearance of "nitrate reaction" in 0·02 % nitrate peptone water.)

Date	Bacteria per c.c. Gelatin at 22° C.	Bacteria per c.c. Agar at 37° C.	*B. coli* present in fraction of 1 c.c.	*Streptococci* present in fraction of 1 c.c.	Denitrifying bacteria
Oct. 4, '05	1,000,000	750,000	1/100,000	1/10,000	1/10,000
,, 12	1,000,000	140,000	1/100,000	1/100,000	1/10,000
,, 30	2,700,000	210,000	1/100,000	—	1/10,000
Dec. 12	—	—	1/10,000	1/10,000	1/10,000
	(Gas formation in 0·25 % nitrate broth.)				
Nov. 17	6,500,000	1,100,000	1/100,000	1/10,000	1/10,000
Dec. 12	—	—	—	—	1/1000
Average	2,800,000	550,000	410,000	162,000	100,000 per c.c.

Sprinkler Effluent.

(By "nitrate reaction" in 0·02 % nitrate peptone water.)

Oct. 4	(less than 100,000)	(less than 10,000)	(less than 1000)	1/1000	1/100
,, 12	70,000	10,000	1/1000	1/10,000	1/10
,, 30	200,000	26,000	1/1000	—	1/1000
Dec. 12	—	—	1/1000	1/1000	1/1000
	(By gas formation in 0·25 % nitrate broth.)				
Nov. 17	460,000	170,000	1/1000	1/1000	1/10
Dec. 12	—	—	—	—	(less than 10)
Average	195,000	52,000	4,000	16,000	1,700 per c.c.

Effluent from Upper Denitrifying Bed.

	(By gas formation in 0·25 % nitrate broth.)				
Nov. 17	2,500,000	710,000	1/10,000	1/10,000	1/1000
	(By "nitrate reaction" in 0·02 % nitrate peptone water 1/1000.)				
Dec. 12	—	—	1/10,000	1/10,000	1/100

Effluent from Lower Denitrifying Bed.

	(By gas formation in 0·25 % nitrate broth.)				
Nov. 17	2,200,000	600,000	1/10,000	1/10,000	1/10,000
Dec. 12	—	—	1/10,000	1/1000	(less than 100)
	(By "nitrate reaction" in 0·02 % nitrate peptone water.)				
Dec. 12	—	—	—	—	1/1000

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